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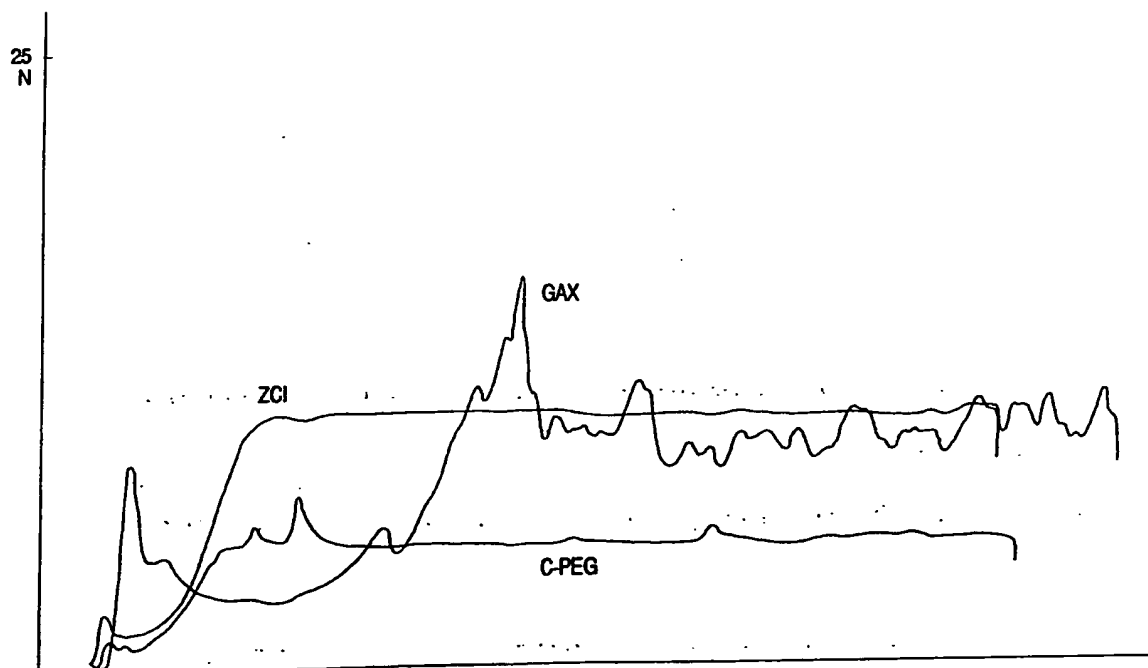
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(54) Title: COLLAGEN-POLYMER CONJUGATES



(57) Abstract

Collagen, particularly atelopeptide collagen, exhibits improved handling characteristics when chemically conjugated and/or crosslinked with a synthetic hydrophilic polymer.

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COLLAGEN-POLYMER CONJUGATES

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DescriptionTechnical Field

This invention relates to proteins and chemically-modified proteins. More specifically, this invention
15 relates to collagen modified by conjugation with synthetic hydrophilic polymers.

Background of the Invention

Collagen is the major protein component of bone,
20 cartilage, skin, and connective tissue in animals. Collagen in its native form is typically a rigid, rod-shaped molecule approximately 300 nm long and 1.5 nm in diameter. It is composed of three collagen polypeptides which form a tight triple helix. The collagen polypep-
25 tides are characterized by a long midsection having the repeating sequence -Gly-X-Y-, where X and Y are often proline or hydroxyproline, bounded at each end by the "telopeptide" regions, which constitute less than about 5% of the molecule. The telopeptide regions of the
30 collagen chains are typically responsible for the cross-linking between chains, and for the immunogenicity of the protein. Collagen occurs in several "types", having differing physical properties. The most abundant types are Types I-III.

35 Collagen is typically isolated from natural sources, such as bovine hide, cartilage, or bones. Bones are usually dried, defatted, crushed, and demineralized

to extract collagen, while hide and cartilage are usually minced and digested with proteolytic enzymes (other than collagenase). As collagen is resistant to most proteolytic enzymes, this procedure conveniently serves to
5 remove most of the contaminating protein found with collagen.

Collagen may be denatured by boiling, which produces the familiar product gelatin.

Daniels et al, U.S. Pat. No. 3,949,073, disclosed
10 the preparation of soluble collagen by dissolving tissue in aqueous acid, followed by enzymatic digestion. The resulting atelopeptide collagen is soluble, and substantially less immunogenic than unmodified collagen. It may be injected into suitable locations of a subject with a
15 fibril-formation promoter (described as a polymerization promoter in the patent) to form fibrous collagen implants in situ, for augmenting hard or soft tissue. This material is now commercially available from Collagen Corporation (Palo Alto, CA) under the trademark Zyderm®
20 collagen implant.

Luck et al, U.S. Pat. No. 4,488,911, disclosed a method for preparing collagen in solution (CIS), wherein native collagen is extracted from animal tissue in dilute aqueous acid, followed by digestion with an enzyme such
25 as pepsin, trypsin, or Pronase®. The enzyme digestion removes the telopeptide portions of the collagen molecules, providing "atelopeptide" collagen in solution. The atelopeptide CIS so produced is substantially non-immunogenic, and is also substantially non-cross-linked
30 due to loss of the primary crosslinking regions. The CIS may then be precipitated by dialysis in a moderate shear environment to produce collagen fibers which resemble native collagen fibers. The precipitated, reconstituted fibers may additionally be crosslinked using a chemical
35 agent (for example aldehydes such as formaldehyde and glutaraldehyde), or using heat or radiation. The result-

ing products are suitable for use in medical implants due to their biocompatibility and reduced immunogenicity.

Wallace et al, U.S. Pat. No. 4,424,208, disclosed an improved collagen formulation suitable for use in soft tissue augmentation. Wallace's formulation comprises reconstituted fibrillar atelopeptide collagen (for example, Zyderm® collagen) in combination with particulate, crosslinked atelopeptide collagen dispersed in an aqueous medium. The addition of particulate crosslinked collagen improves the implant's persistence, or ability to resist shrinkage following implantation.

Smestad et al, U.S. Pat. No. 4,582,640, disclosed a glutaraldehyde crosslinked atelopeptide CIS preparation (GAX) suitable for use in medical implants. The collagen is crosslinked under conditions favoring intrafiber bonding rather than interfiber bonding, and provides a product with higher persistence than non-cross-linked atelopeptide collagen, and is commercially available from Collagen Corporation under the trademark Zyplast® Implant.

Nguyen et al, U.S. Pat. No. 4,642,117, disclosed a method for reducing the viscosity of atelopeptide CIS by mechanical shearing. Reconstituted collagen fibers are passed through a fine-mesh screen until viscosity is reduced to a practical level for injection.

Nathan et al, U.S. Pat. No. 4,563,350, disclosed osteoinductive bone repair compositions comprising an osteoinductive factor, at least 5% nonreconstituted (afibrillar) collagen, and the remainder reconstituted collagen and/or mineral powder (e.g., hydroxyapatite). CIS may be used for the nonreconstituted collagen, and Zyderm® collagen implant (ZCI) is preferred for the reconstituted collagen component. The material is implanted in bone defects or fractures to speed ingrowth of osteoclasts and promote new bone growth.

Chu, U.S. Pat. No. 4,557,764, disclosed a "second nucleation" collagen precipitate which exhibits a desir-

able malleability and putty-like consistency. Collagen is provided in solution (e.g., at 2-4 mg/mL), and a "first nucleation product" is precipitated by rapid titration and centrifugation. The remaining supernatant (containing the bulk of the original collagen) is then decanted and allowed to stand overnight. The precipitated second nucleation product is collected by centrifugation.

Chu, U.S. Pat. No. 4,689,399, disclosed a collagen membrane preparation, which is prepared by compressing and drying a collagen gel. The resulting product has high tensile strength.

J.A.M. Ramshaw et al, Anal Biochem (1984) 141:361-65, and PCT application W087/04078 disclosed the precipitation of bovine collagen (types I, II, and III) from aqueous PEG solutions, where there is no binding between collagen and PEG.

Werner, U.S. Pat. No. 4,357,274, disclosed a method for improving the durability of sclero protein (e.g., brain meninges) by soaking the degreased tissue in H₂O₂ or PEG for several hours prior to lyophilizing. The resulting modified whole tissue exhibits increased persistence.

Hiroyoshi, U.S. Pat. No. 4,678,468, disclosed the preparation of polysiloxane polymers having an interpenetrating network of water-soluble polymer dispersed within. The water-soluble polymer may be a collagen derivative, and the polymer may additionally include heparin. The polymers are shaped into artificial blood vessel grafts, and are designed to prevent clotting.

Other patents disclose the use of collagen preparations with bone fragments or minerals. For example, Miyata et al, U.S. Pat. No. 4,314,380 disclosed a bone implant prepared by baking animal bone segments, and soaking the baked segments in a solution of atelopeptide collagen. Deibig et al, U.S. Pat. No. 4,192,021 disclosed an implant material which comprises powdered

calcium phosphate in a pasty formulation with a biodegradable polymer (which may be collagen). There are several references in the art to proteins modified by covalent conjugation to polymers, to alter the solubility, antigenicity and biological clearance of the protein. For example, U.S. Pat. No. 4,261,973 disclosed the conjugation of several allergens to PEG or PPG (polypropylene glycol) to reduce the proteins' immunogenicity. U.S. Pat. No. 4,301,144 disclosed the conjugation of hemoglobin with PEG and other polymers to increase the protein's oxygen carrying capability. EPO 98,110 disclosed coupling an enzyme or interferon to a polyoxyethylene-polyoxypropylene (POE-POP) block polymer increases the protein's half-life in serum. U.S. Pat. No. 4,179,337 disclosed conjugating hydrophilic enzymes and insulin to PEG or PPG to reduce immunogenicity. Davis et al, Lancet (1981) 2:281-83 disclosed the enzyme uricase modified by conjugation with PEG to provide uric acid metabolism in serum having a long half-life and low immunogenicity. Nishida et al, J Pharm Pharmacol (1984) 36:354-55 disclosed PEG-uricase conjugates administered orally to chickens, demonstrating decreased serum levels of uric acid. Inada et al, Biochem & Biophys Res Comm (1984) 122:845-50 disclosed lipoprotein lipase conjugation with PEG to render it soluble in organic solvents. Takahashi et al, Biochem & Biophys Res Comm (1984) 121:261-65 disclosed HRP conjugated with PEG to render the enzyme soluble in benzene. Abuchowski et al, Cancer Biochem Biophys (1984) 7:175-86 disclosed that enzymes such as asparaginase, catalase, uricase, arginase, trypsin, superoxide dismutase, adenosine deaminase, phenylalanine ammonia-lyase, and the like, conjugated with PEG exhibit longer half-lives in serum and decreased immunogenicity. However, these references are essentially concerned with modifying the solubility and biological characteristics of proteins administered in low concentrations in aqueous solution.

M. Chvapil et al, J Biomed Mater Res (1969) 3:315-32 disclosed a composition prepared from collagen sponge and a crosslinked ethylene glycol monomethacrylate-ethylene glycol dimethacrylate hydrogel. The collagen sponge was prepared by lyophilizing an aqueous mixture of bovine hide collagen and methylglyoxal (a tanning agent). The sponge-hydrogel composition was prepared by polymerizing ethylene glycol monomethacrylate and ethylene glycol dimethacrylate in the sponge.

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Disclosure of the Invention

We have discovered that formulations containing reconstituted fibrillar atelopeptide collagen in combination with particulate mineral components (useful, e.g., for treating bone defects and fractures) exhibit physical instability with time, and tend to separate into several phases or layers. Further, the handling characteristics of such compositions are not ideal, and the malleability and elasticity of such formulations could be improved.

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We have now invented a new collagen-polymer conjugate which exhibits superior handling and chemical stability characteristics. The collagen, preferably reconstituted atelopeptide collagen, is chemically bonded to a synthetic hydrophilic polymer, preferably polyethylene glycol, to form a new collagen-polymer conjugate.

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The polymer may be monofunctional or polyfunctional, having one end capable of attachment, or two or more ends capable of attachment. When the polymer is polyfunctional, it may be joined to collagen by one or more ends, i.e., the polymer may crosslink collagen molecules. The collagen-polymer conjugates may be used to replace or reinforce soft tissue, and may be used in combination with a suitable particulate material to treat bone defects. These materials are also useful for coating implants (such as catheters and bone implants) to

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reduce immunogenicity and foreign body reactions. Dried collagen-polymer conjugates, cast into a membranous form, may be used to replace or repair damaged skin (e.g., burned skin), nerve sheaths, blood vessels, heart valves, ophthalmic shields and corneal lenticules. These forms may also be used in dental applications (e.g. for guided tissue regeneration).

The crosslinking reaction between the collagen and polymer may be performed in vitro, or a reaction mixture may be injected for crosslinking in situ. At sufficient density, crosslinked collagen-polymer conjugates resemble cartilage, and are useful as substitutes therefor, (e.g. cranial onlay, ear and nose reconstruction, and the like). Polyfunctional polymers may also be used to crosslink collagen molecules to other proteins (e.g., glycosaminoglycans, chondroitin sulfates, fibronectin, and the like), particularly growth factors, for compositions particularly suited for wound healing, osteogenesis, and immune modulation. Such tethering of growth factors to collagen molecules provides an effective slow-release drug delivery system.

Brief Description of the Drawings

Figure 1 depicts the force necessary to extrude three compositions: Zyderm® collagen implant (ZCI), a glutaraldehyde-crosslinked collagen (GAX), and a collagen-PEG conjugate of the invention.

Figure 2 illustrates the results of the experiment conducted in Example 6E, demonstrating the retention of biologically active TGF- β 1 in a crosslinked collagen-dPEG composition.

Modes of Carrying Out The Invention

A. Definitions

The term "collagen" as used herein refers to all forms of collagen, including those which have been processed or otherwise modified. Preferred collagens are

treated to remove the immunogenic telopeptide regions ("atelo peptide collagen"), are soluble, and will have been reconstituted into fibrillar form. Type I collagen is best suited to most applications involving bone or cartilage repair. However, other forms of collagen are also useful in the practice of the invention, and are not excluded from consideration here. Collagen crosslinked using heat, radiation, or chemical agents such as glutaraldehyde may be conjugated with polymers as described herein to form particularly rigid compositions. Collagen crosslinked using glutaraldehyde or other (nonpolymer) linking agents is referred to herein as "GAX", while collagen crosslinked using heat and/or radiation is termed "HRX."

The term "synthetic hydrophilic polymer" as used herein refers to a synthetic polymer having an average molecular weight and composition which renders the polymer essentially water-soluble. Most hydrophilic polymers achieve this property by incorporating a sufficient number of oxygen (or less frequently nitrogen) atoms available for forming hydrogen bonds in aqueous solution. Hydrophilic polymers used herein will generally be polyoxyethylene, polyethylene glycol, polymethylene glycol, polytrimethylene glycols, polyvinylpyrrolidones, or derivatives thereof. The polymers are preferably linear or only slightly branched (i.e., having only about 2-10 significant free ends), and will not be substantially crosslinked. Other suitable polymers include polyoxyethylene-polyoxypropylene block polymers and copolymers. Polyoxyethylene-polyoxypropylene block polymers having an ethylene diamine nucleus (and thus having four ends) are also available and may be used in the practice of the invention. Naturally occurring polymers such as proteins, starch, cellulose, heparin and the like are expressly excluded from the scope of this definition. All suitable polymers will be non-toxic and non-inflammatory when administered subcutaneously, and will pref-

erably be essentially nondegradable in vivo over a period of at least several months. The hydrophilic polymer may increase the hydrophilicity of the collagen, but does not render it water soluble. Presently preferred hydrophilic polymers are mono- and difunctional polyethylene glycols (PEG). Monofunctional PEG has only one reactive hydroxy group, while difunctional PEG preferably has reactive groups at each end. Monofunctional PEG preferably has an average molecular weight between about 300 and about 15,000, more preferably between about 1,900 and about 8,000, and most preferably about 5,000. Difunctional PEG preferably has a molecular weight of about 400 to about 20,000, more preferably about 3,000 to about 10,000. PEG can be rendered monofunctional by forming an alkylene ether at one end. The alkylene ether may be any suitable alkoxy radical having 1-6 carbon atoms, for example, methoxy, ethoxy, propoxy, 2-propoxy, butoxy, hexyloxy, and the like. Methoxy is presently preferred. Difunctional PEG is provided by allowing a reactive hydroxy group at each end of the linear molecule. The reactive groups are preferably at the ends of the polymer, but may be provided along the length thereof. Polyfunctional molecules are capable of crosslinking the compositions of the invention, and may be used to attach biological growth factors to collagen.

The term "chemically conjugated" as used herein means attached through a covalent chemical bond. In the practice of the invention, a synthetic hydrophilic polymer and collagen may be chemically conjugated by using a linking radical, so that the polymer and collagen are each bound to the radical, but not directly to each other. The term "collagen-polymer" refers to collagen chemically conjugated to a synthetic hydrophilic polymer, within the meaning of this invention. Thus, "collagen-PEG" (or "PEG-collagen") denotes a composition of the invention wherein collagen is chemically conjugated to PEG. "Collagen-dPEG" refers to collagen

chemically conjugated to difunctional PEG, wherein the collagen molecules are typically crosslinked. "Cross-linked collagen" refers to collagen in which collagen molecules are linked by covalent bonds with polyfunctional (including difunctional) polymers. Terms such as "GAX-dPEG" and "HRX-dPEG" indicate collagen crosslinked by both a difunctional hydrophilic polymer and a cross-linking agent such as glutaraldehyde or heat.

Those of ordinary skill in the art will appreciate that synthetic polymers such as polyethyleneglycol cannot practically be prepared having exact molecular weights, and that the term "molecular weight" as used herein refers to the average molecular weight of a number of molecules in any given sample, as commonly used in the art. Thus, a sample of PEG 2,000 might contain polymer molecules ranging in weight from, for example, 1,200 to 2,500 daltons. Specification of a range of molecular weight indicates that the average molecular weight may be any value between the limits specified, and may include molecules outside those limits. Thus, a molecular weight range of about 800 to about 20,000 indicates an average molecular weight of at least about 800, ranging up to about 20 kDa.

The term "available lysine residue" as used herein refers to lysine side chains exposed on the outer surface of collagen molecules, which are positioned in a manner allowing reaction with activated PEG. The number of available lysine residues may be determined by reaction with sodium 2,4,6-trinitrobenzenesulfonate (TNBS).

The terms "treat" and "treatment" as used herein refer to augmentation, repair, prevention, or alleviation of defects, particularly defects due to loss or absence of soft tissue or soft tissue support, or to loss or absence of bone. Additionally, "treat" and "treatment" also refer to the prevention, maintenance, or alleviation of disorders or disease using a biologically active protein coupled to the collagen-polymer composition of

the invention. Accordingly, treatment of soft tissue includes augmentation of soft tissue, for example implantation of collagen-polymer conjugates of the invention to restore normal or desirable dermal contours, as in the removal of dermal creases or furrows, or as in the replacement of subcutaneous fat in maxillary areas where the fat is lost due to aging. Treatment of bone and cartilage includes the use of collagen-polymer conjugates, and particularly collagen-PEG in combination with suitable particulate materials, to replace or repair bone tissue, for example in the treatment of bone non-unions or fractures. Treatment of bone also includes use of cartilaginous collagen-dPEG compositions, with or without additional bone growth factors. Compositions comprising collagen-polymer with ceramic particles, preferably hydroxyapatite and/or tricalcium phosphate, are particularly useful for the repair of stress-bearing bone due to its high tensile strength. Compositions of the invention may additionally include biologically active factors to aid in healing or regrowth of normal tissue. For example, one may incorporate factors such as epidermal growth factor (EGF), transforming growth factor (TGF) alpha, TGF-beta (including any combination of TGF-beta1, TGF-beta2, platelet derived growth factor (PDGF-AA, PDGF-AB, PDGF-BB), acidic fibroblast growth factor (FGF), basic FGF, connective tissue activating peptides (CTAP), B-thromboglobulin, insulin-like growth factors, tumor necrosis factors (TNF), interleukins, colony stimulating factors (CSFs), erythropoietin (EPO), nerve growth factor (NGF), interferons (IFN), osteogenic factors, and the like. Incorporation of such factors, and appropriate combinations of factors, can facilitate the regrowth and remodeling of the implant into normal bone tissue, or may be used in the treatment of wounds. Further, one may chemically link the factors to the collagen-polymer composition by employing a suitable amount of polyfunctional polymer molecules during syn-

thesis. The factors may then be attached to the free polymer ends by the same method used to attach PEG to collagen, or by any other suitable method. By tethering factor molecules to the implant, the effective amount of factor is substantially reduced. Dried collagen-PEG compositions having sponge-like characteristics may be prepared as wound dressings, or when incorporated with growth factors or the like, they serve as effective controlled-release drug delivery matrices.

10 The term "effective amount" refers to the amount of composition required in order to obtain the effect desired. Thus, a "tissue growth promoting amount" of a composition containing a growth factor refers to the amount of factor needed in order to stimulate tissue growth to a detectable degree. Tissue, in this context, includes connective tissue, bone, cartilage, epidermis and dermis, blood, and other tissues.

The term "sufficient amount" as used herein is applied to the amount of carrier used in combination with the collagen-polymer conjugates of the invention. A sufficient amount is that amount which when mixed with the conjugate renders it in the physical form desired, for example, injectable solution, injectable suspension, plastic or malleable implant, rigid stress-bearing implant, and so forth.

25 The term "suitable particulate material" as used herein refers to a particulate material which is substantially insoluble in water, which is biocompatible, and which is immiscible with collagen-polymer. The particles of material may be fibrillar, or may range in size from about 1 to 20 μm in diameter and be bead-like or irregular in shape. Exemplary particulate materials include without limitation fibrillar crosslinked collagen, gelatin beads, crosslinked collagen-dPEG particles, polytetrafluoroethylene beads, silicone rubber beads, hydrogel beads, silicon carbide beads, and glass

beads. Presently-preferred particulate materials are hydroxyapatite and tricalcium phosphate.

The term "solid implant" refers to any solid object which is designed for insertion and use within the body, and includes bone and cartilage implants (e.g., artificial joints, retaining pins, cranial plates, and the like, of metal, plastic and/or other materials), breast implants (e.g., silicone gel envelopes, foam forms, and the like), catheters and cannulas intended for long term (beyond about three days) use in place, artificial organs and vessels (e.g., artificial hearts, pancreases, kidneys, blood vessels, and the like), drug delivery devices (including monolithic implants, pumps and controlled release devices such as Alzet® minipumps, steroid pellets for anabolic growth or contraception, and the like), sutures for dermal or internal use, periodontal membranes, ophthalmic shields, corneal lenticules, and the like.

The term "in situ" as used herein means at the place of administration. Thus, the injectable reaction mixture compositions are injected or otherwise applied to a site in need of augmentation, and allowed to crosslink at the site of injection. Suitable sites will generally be intradermal or subcutaneous regions for augmenting dermal support, at the site of bone fractures for wound healing and bone repair, and within sphincter tissue for sphincter augmentation (e.g., for restoration of continence).

The term "aqueous mixture" of collagen includes liquid solutions, suspension, dispersions, colloids, and the like containing collagen and water.

The term "NFC cartilage" as used herein refers to a composition of the invention which resembles cartilage in physical consistency. NFC cartilage is prepared from nonfibrillar collagen (e.g., collagen in solution) and is crosslinked with a hydrophillic polymer, especially using dPEG. As an artifact of the production process or by design, NFC cartilage may contain about 0-20% fibrillar

collagen. NFC cartilage is generally prepared by adding dPEG in acidic solution to an acidic solution of collagen, and allowing conjugation to occur prior to neutralization. The term "NFC-FC cartilage" refers to a composition similar to NFC cartilage, wherein the percentage of fibrillar collagen is about 20-80%. NFC-FC cartilage is generally prepared by adding dPEG in a neutralizing buffer to an acidic solution of collagen. The neutralizing buffer causes collagen fibril formation during the conjugation process. Similarly, "FC cartilage" refers to a composition of the invention which is prepared from fibrillar collagen and a difunctional hydrophilic polymer. FC cartilage may generally be prepared using dPEG and fibrillar collagen in neutral solutions/suspensions.

B. General Method

B.1 Preparation:

In most general terms, a suitable collagen is chemically bonded to a selected synthetic hydrophilic polymer. Suitable collagens include all types, preferably types I, II and III. Collagens may be soluble (for example, commercially available Vitrogen® 100 collagen-in-solution), and may have or omit the telopeptide regions. Preferably, the collagen will be reconstituted fibrillar atelopeptide collagen, for example Zyderm® collagen implant (ZCI) or atelopeptide collagen in solution (CIS). Various forms of collagen are available commercially, or may be prepared by the processes described in, for example, U.S. Pat. Nos. 3,949,073; 4,488,911; 4,424,208; 4,582,640; 4,642,117; 4,557,764; and 4,689,399, all incorporated herein by reference.

The compositions of the invention comprise collagen chemically conjugated to a selected synthetic hydrophilic polymer or polymers. Collagen contains a number of available amino and hydroxy groups which may be used to bind the synthetic hydrophilic polymer. The polymer may

be bound using a "linking group", as the native hydroxy or amino groups in collagen and in the polymer frequently require activation before they can be linked. For example, one may employ compounds such as dicarboxylic anhydrides (e.g., glutaric or succinic anhydride) to form a polymer derivative (e.g., succinate), which may then be activated by esterification with a convenient leaving group, for example, N-hydroxysuccinimide, N,N'-disuccinimidyl oxalate, N,N'-disuccinimidyl carbonate, and the like. See also Davis, U.S. Pat. No. 4,179,337 for additional linking groups. Presently preferred dicarboxylic anhydrides that are used to form polymer-glutarate compositions include glutaric anhydride, adipic anhydride, 1,8-naphthalene dicarboxylic anhydride, and 1,4,5,8-naphthalenetetracarboxylic dianhydride. The polymer thus activated is then allowed to react with the collagen, forming a collagen-polymer composition of the invention.

In a preferred embodiment, monomethylpolyethylene glycol (mPEG) (mw 5,000) is reacted with glutaric anhydride to form mPEG glutarate. The glutarate derivative is then reacted with N-hydroxysuccinimide to form a succinimidyl monomethylpolyethylene glycol glutarate. The succinimidyl ester (mPEG*, denoting the activated PEG intermediate) is then capable of reacting with free amino groups present on collagen (lysine residues) to form a collagen-PEG conjugate of the invention wherein one end of the PEG molecule is free or nonbound. Other polymers may be substituted for the monomethyl PEG, as described above. Similarly, the coupling reaction may be carried out using any known method for derivatizing proteins and synthetic polymers. The number of available lysines conjugated may vary from a single residue to 100% of the lysines, preferably 10%-50%, and more preferably 20-30%. The number of reactive lysine residues may be determined by standard methods, for example by reaction with TNBS.

The resulting product is a smooth, pliable, rubbery mass having a shiny appearance. It may be wetted, but is

not water-soluble. It may be formulated as a suspension at any convenient concentration, preferably about 30-65 mg/mL, and may be implanted by injection through a suitable syringe. The consistency of the formulation may be
5 adjusted by varying the amount of liquid used.

Formulations suitable for repair of bone defects or nonunions may be prepared by providing high concentration compositions of collagen-polymer, or by admixture with
10 suitable particulate materials. Such collagen-polymer particulate compositions may be malleable or rigid, depending on the amount of liquid incorporated. Formulations for treatment of stress-bearing bone is preferably dried and rigid, and will generally comprise between about 45% and 85% particulate mineral, for
15 example hydroxyapatite or tricalcium phosphate. The tensile strength and rigidity may be further increased by heating the composition under vacuum at about 60-90°C, preferably about 75°C, for about 5 to 15 hours, preferably about 10 hours. Malleable compositions may be
20 used for repair of non-stressed bone.

The activated mPEG* may be replaced, in whole or in part, by difunctional activated PEG (dPEG*, e.g., non-methylated PEG which is then activated at each end), thus providing a crosslinked or partially crosslinked collagen
25 composition. Such compositions are, however, quite distinct from conventionally-crosslinked collagen compositions (e.g., using heat, radiation, glutaraldehyde, glycosaminoglycans and the like), as the long-chain synthetic hydrophilic polymer imparts a substantial
30 hydrophilic character to the composition. In a presently preferred embodiment, approximately 1-20% of the mPEG is difunctional PEG. The character of the composition may be adjusted as desired, by varying the amount of difunctional PEG included during the process.

35 In another presently preferred embodiment, difunctional PEG* (substantially 100% at pH 7) is used to crosslink collagen. In one version, CIS (about 3-100

mg/mL, preferably about 10-40 mg/mL) is allowed to react with dPEG* (difunctional PEG activated at each end by addition of an acid anhydride having a leaving group such as succinimide) having a molecular weight of about 2,000 to about 20,000 (preferably about 3,400-10,000) which is added as a concentrated solution to a final reaction mixture concentration of about 5-40%, preferably about 10-20%. This represents a 5- to 10-fold excess of dPEG* to collagen on a molar basis. The collagen molecules bind to dPEG*, without mechanical mixing or agitation, and settle out of solution to produce a cartilaginous collagen-polymer conjugate containing approximately 20-80% fibrillar collagen. The conjugate is then washed with PBS to remove any remaining unreacted dPEG*, providing the material of the invention. A cartilaginous collagen-polymer conjugate may also be prepared by mixing dPEG* solution (pH 3) with collagen-in-solution between two syringes to homogeneity, and then casting into a suitable container (e.g., a Petri dish). A 20% w/v dPEG* solution (pH 7) is then added to the non-fibrillar collagen-PEG solution to result in a lightly cartilaginous fibrillar collagen-polymer conjugate. The resulting NFC-FC conjugate cartilage contains approximately 1-40% fibrillar collagen. The characteristics of the final product may be adjusted by varying the initial reaction conditions. In general, increased collagen and/or polymer concentrations provide a denser, less porous product. By varying the pH of the collagen solution and the dPEG* solution, compositions may be produced over a wide range of fibrillar content. If desired, the denser formulations may be cast or molded into any shape desired, for example into sheets or membranes, into tubes or cylinders, into cords or ropes, and the like.

A particulate microgel material may be achieved by agitating a reaction mixture of collagen and dPEG* during crosslinking (e.g., by stirring or passing between syringes). Such materials are smooth, pliable, rubbery

masses, with a shiny appearance, however, they have higher tensile strength than collagen-mPEG conjugates or glutaraldehyde chemically crosslinked collagen that is not conjugated to a polymer. The injectable formulations (gels or solutions) may be used to dip coat implants, catheters, tubes (e.g., for vein replacement), meshes (e.g., for tissue reinforcement) and the like. Gels may be prepared by reducing the polymer concentration or reducing the reaction time. CIS is the preferred starting material where the desired properties are high density, rigidity, viscosity, and translucence. However, one may substitute fibrillar collagen (preferably atelopeptide fibrillar collagen such as ZCI) and obtain products which are more opaque, more flexible, and more susceptible to colonization by cells after implantation. CIS-based materials are presently preferred for coating articles to be implanted, such as catheters and stress-bearing bone implants. Fibrillar collagen-based materials are preferred for applications such as dermal augmentation, sphincter augmentation, resurfacing of eroded joint surfaces (as in rheumatoid arthritis), replacement of tendons and ligaments, and preparation of artificial vessels (e.g., veins).

Compositions of the invention containing biological growth factors such as EGF and TGF- β are prepared by mixing an appropriate amount of the factor into the composition, or by incorporating the factor into the collagen prior to treatment with activated PEG. By employing an appropriate amount of difunctional PEG, a degree of crosslinking may be established, along with molecules consisting of collagen linked to a factor by a synthetic hydrophilic polymer. Preferably, the factor is first reacted with a molar excess of dPEG* in a dilute solution over a 3 to 4 hour period. The factor is preferably provided at a concentration of about 1 $\mu\text{g/mL}$ to about 5 mg/mL , while the dPEG* is preferably added to a final concentration providing a 30 to 50-fold molar

excess. The resulting conjugated factor is then added to an aqueous collagen mixture (about 1 to about 60 mg/mL) at pH 7-8 and allowed to react further. The resulting composition is allowed to stand overnight at ambient temperature. The pellet is collected by centrifugation, and is washed with PBS by vigorous vortexing in order to remove non-bound factor.

Flexible sheets or membranous forms of the collagen-polymer conjugate may be prepared by methods known in the art, for example, U.S. Patent Nos. 4,600,533; 4,412,947; and 4,242,291. Briefly, high concentration (10-100 mg/mL) CIS or fibrillar collagen (preferably atelopeptide fibrillar collagen, such as ZCI) is cast into a flat sheet container. A solution of mPEG* (having a molecular weight of approximately 5,000) is added to the cast collagen solution, and allowed to react overnight at room temperature. The resulting collagen-polymer conjugate is removed from the reaction solution using a sterile spatula or the like, and washed with PBS to remove excess unreacted mPEG*.

The resulting conjugate may then be compressed under constant pressure to form a uniform, flat sheet or mat, which is then dried to form a membranous implant of the invention. More flexible membranous forms are achieved by using lower collagen concentrations and high polymer concentrations as starting materials.

Less flexible membranous forms are prepared by using a dPEG* solution rather than mPEG*. CIS, at room temperature, is mixed with a buffer solution and incubated at 37°C overnight. The resulting gel is compressed under constant pressure, dried, and desalted by washing. The resultant membrane is then crosslinked by treating with dPEG*, washed, and then dried at low temperature.

Collagen-polymer conjugates may also be prepared in the form of sponges, by lyophilizing an aqueous slurry of the composition after conjugation.

Alternatively, CIS or fibrillar collagen (10-100 mg/mL) is cast into a flat sheet container. A solution of dPEG* (22-50% w/v) is added to the cast collagen. The mixture is allowed to react over several hours at room temperature. Shorter reaction times result in more flexible membranes. The resulting collagen-polymer membrane may be optionally dehydrated under a vacuum oven, lyophilization, or air-drying.

B.2 Use and Administration:

Compositions of the invention have a variety of uses. Malleable, plastic compositions may be prepared as injectable formulations, and are suitable for dermal augmentation, for example for filling in dermal creases, and providing support for skin surfaces. Such compositions are also useful for augmenting sphincter tissue, (e.g., for restoration of continence). In such cases, the formulation may be injected directly into the sphincter tissue to increase bulk and permit the occluding tissues to meet more easily and efficiently. These compositions may be homogeneous, or may be prepared as suspensions of small microgel collagen-polymer conjugate particles or beads.

Surprisingly, one may administer the reaction mixture by injection before crosslinking has completed. In this embodiment, an aqueous collagen mixture is combined with a low-concentration dPEG* solution, mixed, and the combination injected or applied before the viscosity increases sufficiently to render injection difficult (usually about 20 minutes). Mixing may be accomplished by passing the mixture between two syringes equipped with Luer lock hubs, or through a single syringe having dual compartments (e.g., double barrel). The composition crosslinks in situ, and may additionally crosslink to the endogenous tissue, anchoring the implant in place. In this method, one can use collagen (preferably fibrillar collagen) at a concentration of about 10-100 mg/mL, although about 30-80 mg/mL is preferred, most preferably

about 33 mg/mL. The dPEG* concentration is preferably set at about 0.1 to about 3%, although concentrations as high as 30% may be used if desired. The mixture is injected directly into the site in need of augmentation, and causes essentially no detectable inflammation or foreign body reaction. One may additionally include particulate materials in the collagen reaction mixture, for example hydrogel or collagen-dPEG beads, or hydroxyapatite/tricalcium phosphate particles, to provide a bulkier or more rigid implant after crosslinking.

Compositions of the invention (particularly cross-linked collagen compositions) are also useful for coating articles for implantation or relatively long term residence within the body. Such surface treatment renders the object nonimmunogenic, and reduces the incidence of foreign body reactions. Accordingly, one can apply compositions of the invention to catheters, cannulas, bone prostheses, cartilage replacement, breast implants, minipumps and other drug delivery devices, artificial organs, and the like. Application may be accomplished by dipping the object into the reaction mixture while crosslinking is occurring, and allowing the adherent viscous coating to dry. One may pour or otherwise apply the reaction mixture if dipping is not convenient. Alternatively, one may use flexible sheets or membranous forms of collagen-polymer conjugate to wrap the object with, sealing corners and edges with reaction mixture.

In another embodiment, the object may be dipped in a viscous collagen-in-solution bath, or in a fibrillar collagen solution until the object is completely coated. The collagen solution is fixed to the object by dipping the collagen-coated object into a dPEG* (pH 7) solution bath, and then allowing the collagen-polymer coated object to dry. Alternatively, viscous collagen-in-solution is mixed with a dPEG* (pH 3) solution and polymerized rapidly, as described above. The object is

dipped in the acidic collagen-polymer solution, and cured by dipping the coated object into a neutralizing buffer containing about 20% by weight dPEG* (pH 7), to result in a collagen-polymer coated object.

5 Compositions of the invention may be prepared in a form that is dense and rigid enough to substitute for cartilage. These compositions are useful for repairing and supporting tissue which require some degree of structure, for example in reconstruction of the nose, ear,
10 knee, larynx, tracheal rings, and joint surfaces. One can also replace tendon, ligament and blood vessel tissue using appropriately formed cartilaginous material. In these applications, the material is generally cast or molded into shape: in the case of tendons and liga-
15 ments, it may be preferable to form filaments for weaving into cords or ropes. In the case of artificial blood vessels it may be advantageous to incorporate a reinforcing mesh (e.g., nylon or the like).

 Compositions of the invention which contain growth
20 factors are particularly suited for sustained administration of factors, as in the case of wound healing promotion. Osteoinductive factors and cofactors (including TGF- β) may advantageously be incorporated into compositions destined for bone replacement, augmentation,
25 and/or defect repair. Compositions provided in the form of a membrane may be used to wrap or coat transplanted organs, to suppress rejection and induce improved tissue growth. Similarly, one may dip coat organs for transplantation using a crosslinking reaction mixture of
30 factor-polymer conjugates and collagen. Alternatively, one may administer antiviral and antitumor factors such as TNF, interferons, CSFs, TGF- β , and the like for their pharmaceutical activities. The amount of composition used will depend upon the severity of the condition being
35 treated, the amount of factor incorporated in the composition, the rate of delivery desired, and the like. However, these parameters may easily be determined by

routine experimentation, for example by preparing a model composition following the examples below, and assaying the release rate in a suitable animal model.

5 C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

10

Example 1

(Preparation of Collagen-PEG)

(A) Monomethyl-PEG 5000 (50 g, 10 mmol, Aldrich Chemical Co.) is dissolved in 1,2-dichloroethane (250 mL) and heated at reflux with glutaric anhydride (5 g) and pyridine (4 mL) under nitrogen for 3 days. The solution is then filtered and the solvent evaporated, and the residue dissolved in water (100 mL) and washed with diethyl ether (2X 50 mL). The resulting PEG-glutarate is extracted from the water with chloroform (2X 50 mL), and the chloroform evaporated to yield about 43 g of PEG-glutarate. The PEG-glutarate is then dissolved in dimethylformamide (DMF, 200 mL) at 37°C, and N-hydroxysuccinimide (10% molar xs) added. The solution is cooled to 0°C, and an equivalent amount of dicyclohexylcarbodiimide added in DMF solution (10 mL). The mixture is left at room temperature for 24 hours, and then filtered. Cold benzene (100 mL) is then added, and the PEG-succinimidyl glutarate (PEG-SG) precipitated by adding petroleum ether (200 mL) at 0°C. The precipitate is collected on a sintered glass filter. Dissolution in benzene, followed by precipitation with petroleum ether is repeated three times to provide "activated" PEG (PEG-SG).

35 Vitrogen 100® collagen in solution (400 mL, 1.2 g collagen, 0.004 mmol) was mixed with 0.2 M phosphate buffer (44 mL) to elevate the pH to 7.4. Next, a three-

fold molar excess of PEG-SG (6.00 g, 1.2 mmol) was dissolved in water for injection (40 mL) and sterile-filtered. The PEG-SG solution was then added to the collagen solution, and the mixture allowed to stand at 17-22°C for about 15 hours. The solution was then centrifuged, and the resulting pellet (25 g) of reconstituted fibrils collected and washed with phosphate-buffered saline (PBS, 3X 400 mL) to remove residual PEG. The resulting material has a solid, coherent elasticity, and may be picked up on a spatula (the equivalent non-conjugated collagen, Zyderm® collagen implant is more fluid). The resulting material may be diluted with PBS to provide a dispersion having 20.5 mg/mL collagen-PEG.

(B) Similarly, proceeding as in part (A) above but substituting polypropylene glycol and POE-POP block polymers for polyethylene glycol, the corresponding collagen-PPG and collagen-POE-POP compositions are prepared.

(C) Difunctional PEG 3400 (34 g, 10 mmol, Aldrich Chemical Co.) is dissolved in 1,2-dichloroethane (250 mL) and heated at reflux with glutaric anhydride (10 g) and pyridine (4 mL) under nitrogen for 3 days. The solution is then filtered and the solvent evaporated, and the residue dissolved in water (100 mL) and washed with diethyl ether (2X 50 mL). The resulting PEG-diglutarate is extracted from the water with chloroform (2X 50 mL), and the chloroform evaporated to yield PEG-diglutarate. The PEG-diglutarate is then dissolved in DMF (200 mL) at 37°C, and N-hydroxysuccinimide (10% molar xs) added. The solution is cooled to 0°C, and an equivalent amount of dicyclohexylcarbodiimide added in DMF solution (10 mL). The mixture is left at room temperature for 24 hours, and then filtered. Cold benzene (100 mL) is then added, and the PEG-di(succinimidyl glutarate) (dPEG-SG) precipitated by adding petroleum ether (200 mL) at 0°C. The precipitate is collected on a sintered glass filter. Dissolution in benzene, followed by precipitation with petroleum

ether is repeated three times to provide "activated" dPEG (dPEG*).

Vitrogen 100® collagen in solution (400 mL, 1.2 g collagen, 0.004 mmol) was mixed with 0.2 M phosphate buffer (44 mL) to elevate the pH to 7.4. Next, a three-fold molar excess of dPEG* (6.00 g, 1.2 mmol) was dissolved in water for injection (40 mL) and sterile-filtered. The dPEG* solution was then added to the collagen solution, agitated, and the mixture allowed to stand at 17-22°C for about 15 hours. The solution was then centrifuged, and the resulting pellet of reconstituted fibrils collected and washed with PBS (3X 400 mL) to remove residual dPEG*. The pellet was then placed in a syringe fitted with a Luer lock hub connected to a second syringe, and was passed between the syringes until homogeneous. The resulting material is a microgel or a particulate suspension of random size fibrils in solution (microgel conjugate). The material is a smooth, pliable, rubbery mass, with a shiny appearance.

(D) Preparation of Cartilaginoid Conjugates:

Approximately 20% by weight of dPEG* (pH 7) was added to collagen in solution (33.8 mg/mL), and incubated at 21°C for about 16 hours. The resulting conjugate was washed with 100 mL PBS 3-5 times over 12 hours. The resulting cartilaginoid non-fibrillar collagen-polymer conjugate (NFC-FC cartilage) was a translucent solid with coherent elasticity. The product contained approximately 20-80% fibrillar collagen.

Another NFC cartilage composition was prepared by mixing dPEG* solution (0.6 g, pH 3) with collagen in solution (33.8 mg/mL, pH 2). The mixture was passed between two syringes joined by a Luer lock connector to form a homogenous solution. A solution of dPEG* (20% w/v) in a neutralizing buffer was then added to result in a substantially non-fibrillar collagen (NFC) cartilage material. The resulting product contained approximately 1-40% fibrillar collagen.

Alternatively, fibrillar collagen may be used instead of CIS to produce a cartilaginous fibrillar collagen-polymer conjugate (FC cartilage) having an opaque appearance and high fibrillar content. Such FC
5 cartilage is more porous and permeable than non-fibrillar collagen-polymer conjugates.

Example 2

(Characterization)

10 (A) Collagen-mPEG prepared in Example 1A was characterized and compared with Zyderm® collagen implant (ZCI), and glutaraldehyde-crosslinked fibrillar collagen (GAX).

Extrusion:

15 This assay measured the force required to extrude the test composition through a 30 gauge needle. The results are shown in Figure 1. As can be seen from the graph of force required (in Newtons) versus plunger travel, ZCI was extruded smoothly, requiring a force of
20 about 20-30 Newtons. GAX was not extruded smoothly, as shown by the "spiking" exhibited in the force trace. At the plateau, GAX required about 10-15 N for extrusion. In contrast, collagen-mPEG demonstrated a very low extrusion force (8-10 N), with little or no spiking.

25 Intrusion:

Intrusion is a measure of the tendency of a composition to "finger" or channel into a porous bed, rather than remaining in a compact mass. Low intrusion is preferred in augmentation of soft tissue, so that the
30 injected implant does not diffuse through the dermis and remains in place.

A 1 mL syringe fitted with a 30 gauge needle was half-filled with silicon carbide particles (60 mesh), simulating human dermis. The upper half of the syringe
35 was filled with 0.5 mL test composition (GAX, ZCI, or collagen-mPEG) at 35 mg/mL. The plunger was then fitted, and depressed. On depression, ZCI appeared at the

needle, demonstrating intrusion through the silicon carbide bed. Syringes filled with GAX or collagen-mPEG of the invention did not pass collagen, instead releasing only buffer, demonstrating no intrudability.

5 Helicity:

The portion of each composition exhibiting non-helical character was measured using sensitivity to digestion with trypsin. Samples were treated with the protease trypsin, which is capable of attacking only
10 fragmented portions of the collagen protein. The extent of hydrolysis is measured by fluorescamine assay for solubilized peptides, and the results are expressed as percentage non-helical collagen. The percentage of non-helical collagen was measured 30 minutes after the begin-
15 ning of the digestion period. The results indicated that ZCI was 3-10% sensitive, GAX was 1-2% sensitive, and collagen-mPEG was about 1% sensitive. Sensitivity to trypsin may also correlate to sensitivity to endogenous proteases following implantation.

20 Collagenase Sensitivity:

The sensitivity of each composition to collagenase was also measured. ZCI was 65.2% digested, compared to 2.2% for GAX, and 45.8% for collagen-mPEG.

Phase Transition:

25 The behavior of each composition vs. temperature was examined using a differential scanning calorimeter. On heating, ZCI exhibited multiple peaks at about 45 and 53°C. GAX exhibited a peak at 67-70°C. Collagen-mPEG exhibited a peak at 56-61°C.

30 Lysine Content:

The number of free lysines per mole was determined for each composition using TNBS to quantify reactive epsilon amino groups. ZCI exhibited about 30 lysines per (single helix) molecule (K/m), whereas GAX exhibited 26-
35 27 K/m, and collagen-mPEG 21-26 K/m.

(B) Characterization of Crosslinked Collagen-Polymer Conjugates:

A collagen-dPEG conjugate prepared as described in Example 1C was characterized using differential scanning calorimetry (DSC). This test is a measure of the transition temperature during fragmentation of the collagen molecule at a microscopic level. A lowering of the transition temperature indicates an increase in fragmentation in a manner similar to that measured by trypsin sensitivity.

The collagen-dPEG conjugate showed a single denaturational transition at 56°C by DSC, which is similar to the typical melting point of the Collagen-PEG conjugate prepared in Example 1A. In comparison, ZCI has a melting temperature of 45-53°C with multiple denaturational transitions, and GAX has a melting temperature of 67-70°C with a single denaturational transition.

The extrusion test described in Example 2A could not be used to characterize the collagen-dPEG conjugate because the material was not extrudable through a 30 gauge needle.

Using the intrusion test described in Example 2A, the passage of collagen-dPEG was completely blocked at the silicon carbide bed, which indicates high crosslinking between the collagen molecules and little or no intrudability.

Example 3

(Immunogenicity)

30 (A) Non-crosslinked PEG-Collagen:

This experiment was conducted to demonstrate the relative immunogenicity of a collagen-mPEG preparation of the invention versus a commercially-available bovine collagen formulation prepared from essentially the same source material, and having a similar consistency. As both collagen preparations were prepared using atelo-peptide collagen (which is only weakly immunogenic), the

preparations were formulated with either complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA), to enhance the immune response. This is a severe test, designed to magnify any possible immune reaction.

5 Collagen-mPEG was prepared as in Example 1A above. Male Hartley guinea pigs (11) were anesthetized and bled by heart puncture for pre-immunization serologic evaluation. Five animals were treated with two 0.1 mL intramuscular injections of Zyderm® collagen implant (ZCI) emulsified in CFA (1:9) in the left and right thighs. 10 Another five animals were treated in the same fashion, using collagen-PEG (35 mg/mL) emulsified in CFA. One animal was treated with collagen-PEG in IFA. At day 14 following immunization, all animals were again bled by 15 heart puncture, and serum obtained for antibody titer determination (using ELISA). Serology was again performed at day 30.

On day 30, following collection of serum samples, each animal was challenged intradermally with both ZCI 20 and collagen-PEG (0.1 mL of each, one on each flank). Delayed-type hypersensitivity (DTH) was quantified as a measure of cell-mediated immunity. DTH was evaluated at 24, 48, and 72 hours post-challenge by measuring the diameter of any wheal using micrometer calipers, and 25 noting the extent of erythema and induration. Animals were then euthanized with CO₂, and the injection sites excised and fixed in neutral, buffered formalin for histological study.

Serological results indicated reduced immuno- 30 genicity of collagen-PEG vs. ZCI. At day 14, 80% of ZCI immunized animals exhibited "positive" antibody responses (titer \geq 160 at day 14), whereas 0% of the collagen-PEG immunized animals exhibited positive responses. At day 30, all ZCI-immunized animals exhibited high antibody 35 titers, whereas none of the collagen-PEG-immunized animals (C-PEG) exhibited high titers. The data are shown in Table 1.

Table 1: Immunogenicity

| 5 | Animal | Treatment | Antibody Titer | |
|----|--------|-------------|----------------|--------|
| | | | day 14 | day 30 |
| | 1 | ZCI | 320 | >2560 |
| | 2 | ZCI | 320 | 1280 |
| | 3 | ZCI | 2560 | >2560 |
| 10 | 4 | ZCI | 320 | >2560 |
| | 5 | ZCI | 80 | 2560 |
| | 6 | C-PEG | 0 | 0 |
| | 7 | C-PEG | 0 | 160 |
| | 8 | C-PEG | 40 | 640 |
| 15 | 9 | C-PEG | 0 | 20 |
| | 10 | C-PEG | 0 | 640 |
| | 11 | C-PEG (IFA) | 0 | 160 |

Responses to the DTH challenge also demonstrated
 20 that the collagen-mPEG of the invention is less immuno-
 genic. Guinea pigs immunized with ZCI and challenged
 with ZCI exhibited a wheal measuring 1.128 ± 0.058 cm in
 diameter. Animals immunized with collagen-mPEG and chal-
 25 lenged with collagen-mPEG exhibited wheals measuring
 0.768 ± 0.036 cm. Animals immunized with ZCI and chal-
 lenged with collagen-mPEG, or immunized with collagen-
 mPEG and challenged with ZCI, developed wheals smaller
 than the ZCI-immunized ZCI-challenged wheals. Responses
 measured at 48 and 72 hours were essentially the same or
 30 lower than the 24 hour response for each site. Erythema
 was essentially the same for all animals.

Histological studies showed that both materials
 exhibited comparable intrusion, fingering into the dermis
 and subcutaneous space. Sites of intradermal challenge
 35 with ZCI in ZCI-immunized animals exhibited the most
 extensive inflammatory response, including a cellular
 infiltrate of lymphohistiocytic elements with eosinophils
 and occasional giant cells. Two of the implant sites
 demonstrated an erosive inflammation of the overlying
 40 epidermis and eschar formation. Sites of intradermal
 challenge with collagen-mPEG in ZCI-immunized animals
 exhibited only a moderate associated inflammatory infil-

trate, with a marked reduction in acute cells and lymphoid elements. Histiocytes and giant cells were more prevalent, and in some samples lined and colonized the implants heavily. Animals immunized with collagen-mPEG exhibited only slight to moderate reaction, with ZCI challenge sites accompanied by a modest lymphohistiocytic perivascular infiltrate with a few eosinophils and giant cells. Collagen-mPEG challenge sites were typically accompanied by a minimal scattering of lymphoid cells near the associated vasculature.

(B) Crosslinked dPEG-Collagen Conjugates:

Collagen-dPEG conjugates were prepared as in Example 1D. The samples were implanted in the dorsal subcutis and as cranial onlays in rats. After implantation for 30 days in the subcutis, NFC cartilage and NFC-FC cartilage materials had a homogeneous microfibrillar structure. Mild colonization by connective tissue cells occurred at the periphery of the NFC-FC cartilage samples, and mild capsule formation was present. No colonization had occurred with the NFC cartilage material and mild capsule formation was present. FC cartilage had a very fibrous structure with mild but frequently deep colonization by connective tissue cells and sparse numbers of adipocytes. Trace amounts of capsule were present in limited areas of the FC cartilage samples. NFC cartilage materials tended to retain their pre-implantation shape, with sharply defined edges, while the NFC-FC cartilage samples tended to flatten over time and develop rounded profiles.

When implanted as cranial onlays, the appearance of each of the materials was similar to that in the subcutis except that the samples tended to become anchored to the skull via integration of the capsule or surrounding loose connective tissue with the periosteum.

All of the samples appeared to be biocompatible, have differing degrees of colonization by host tissues, and varying mechanical characteristics.

Example 4(In situ Crosslinking)

A dPEG solution was prepared as described in Example 1C above. The following samples were then prepared:

- (1) 5 mg dPEG in 80 μ L water, mixed with 0.5 mL fibrillar collagen (35 mg/mL), to a final dPEG concentration of 1% by volume;
- 10 (2) 15 mg dPEG in 80 μ L water, mixed with 0.5 mL fibrillar collagen (35 mg/mL), to a final dPEG concentration of 3% by volume;
- (3) Vitrogen® 100 collagen in solution;
- 15 (4) 5 mg dPEG in 80 μ L water, mixed with 0.5 mL non-fibrillar collagen (35 mg/mL), to a final dPEG concentration of 1% by volume;
- (5) 15 mg dPEG in 80 μ L water, mixed with 0.5 mL non-fibrillar collagen (35 mg/mL), to a final dPEG concentration of 3% by volume;
- 20 (6) 5 mg dPEG in 0.5 mL PBS, to a final dPEG concentration of 1% by volume; and
- (7) GAX.

The dPEG solutions of Samples 1, 2, 4, and 5 were placed in a 1 mL syringe equipped with a Luer lock fitting and connector, and joined to another syringe containing the collagen material. The solutions were mixed by passing the liquids back and forth between the syringes several times to form the homogeneous reaction mixture.

30 The syringe connector was then removed and replaced with a 27 gauge needle, and approximately 50 μ L of the reaction mixture was injected intradermally into each of 20 guinea pigs. Samples 3, 6, and 7 were similarly administered through a 27 gauge needle. At intervals up to 30 days following injection, the treatment sites were harvested and studied histologically.

By 30 days, all of the materials appeared to be biocompatible. Samples 1 and 2 displayed wide dispersion with an intermediate degree of interdigitation with dermal collagen fibers. Colonization by connective tissue cells was moderate, and a trace of round cell infiltrate with eosinophils was seen.

Samples 3, 4 and 5 were highly dispersed and finely interdigitated with dermal collagen fibers. Colonization was mild to moderate, and trace levels of round cell infiltration were seen.

Sample 6 had no detectable effects. Sample 7 occurred as large islands with moderate colonization and trace to mild levels of inflammation.

15

Example 5

(Coating of Implants)

A collagen-dPEG reaction mixture was prepared as described in Example 1C above. A titanium implant was dipped into the reaction mixture approximately 20 minutes after crosslinking was initiated. The implant was then allowed to finish crosslinking, and dry overnight.

Example 6

(Collagen-Polymer-Growth Factor Conjugates)

25 (A) A conjugate containing crosslinked collagen-dPEG-TGF-B1 was prepared as follows:

A solution of TGF-B1 and ^{125}I -TGF-B1 (10^5 cpm; 25 μL of 1 mg/mL) was added to a solution of dPEG* (4 mg) in CH_2Cl_2 (100 μL), and the mixture allowed to react for 12 (sample #3) or 35 (sample #5) minutes at 17°C . To this was added 2.5 mL of collagen solution (3 mg/mL atelopeptide nonfibrillar collagen), and the resulting mixture allowed to incubate overnight at ambient temperature. The pellet which formed was collected by centrifugation to provide collagen-dPEG-TGF-B1.

35

(B) A composition based on fibrillar atelopeptide collagen was prepared as in part A above, but limit-

ing TGF- β 1/dPEG* reaction time to 2 minutes, and substituting 7 mg of fibrillar collagen (precipitated from collagen in solution within 2 minutes prior to use) for collagen in solution.

- 5 (C) A composition containing dPEG-crosslinked collagen and free TGF- β 1 was prepared as follows:

A solution of dPEG* (4 mg) in CH_2Cl_2 (100 μL), was added to 2.5 mL of CIS (3 mg/mL atelopeptide nonfibrillar collagen), and the resulting mixture allowed to
10 incubate overnight at ambient temperature. The pellet which formed was washed to remove unreacted dPEG*, and 25 μg of TGF- β 1 mixed in to provide collagen-dPEG + TGF- β 1.

- (D) The degree of TGF- β 1 binding was determined as follows:

15 Each composition prepared in parts A-C above was washed six times with 0.5 mL of buffer (0.02 M phosphate buffer, 0.1% BSA) by vigorous vortexing followed by centrifugation in order to remove non-bound TGF- β 1. The pellet and supernatants were collected at each time of
20 washing, and were counted. Figure 2 demonstrates the release rate of the compositions of part A (open circles) and part B (filled circles) versus the simple mixture prepared in part C (x's), showing the number of counts released as a function wash cycle. As shown in
25 the figure, the TGF- β 1 in the simple mixture is quantitatively released within about 6 washings, while approximately 40% of the TGF- β 1 is retained in the compositions of part B and 50% is retained in the compositions of part A.

- 30 (E) The biological activity of the materials prepared above was assayed as follows:

Compositions prepared according to part A (CIS-dPEG-TGF- β 1) (TGF- β 1/dPEG* reaction time of 12 minutes) and part C (CIS-dPEG + TGF- β 1) were prepared, as well as
35 a control prepared according to part C without TGF- β 1 (CIS-dPEG). The samples were washed in PBS/BSA eight times as described in part D, then washed an additional

three times in fetal bovine serum (Gibco) at 37°C. This washing protocol resulted in visually detectable material loss, so remaining TGF- β 1 content was determined by counting the remaining ^{125}I . TGF- β 1 activity was then
5 assayed by ELISA. The results are shown in Table 2 below.

Table 2: Retention of Biological Activity

| Sample | ^{125}I Counts | remaining TGF- β 1(μg) | O.D. (414 nm) |
|---------------------------|----------------------------|--|------------------|
| CIS-dPEG | 0 | 0 | 0.015 0.015 |
| CIS-dPEG + TGF- β 1 | 2775 | 0.5-1.0 | 0.029 0.035 |
| CIS-dPEG-TGF- β 1 | 42604 | 7.4 | 0.102 0.082 |

The data demonstrates that the TGF- β 1 retained in
25 the compositions of the invention remains in a substantially active form.

Example 7

(Formulations)

30 (A) A formulation suitable for implantation by injection was prepared by suspending collagen-PEG in sterile water for injection, at 35 mg/mL. The characteristics of the resulting formulation are described in Example 2 above.

35 (B) A formulation useful for repair of stress-bearing bone defects (e.g., fractures, nonunions, and the like) may be prepared by mixing collagen-PEG of the invention with a suitable particulate, insoluble component. The insoluble component may be fibrillar cross-
40 linked collagen, gelatin beads, polytetrafluoroethylene beads, silicone rubber beads, hydrogel beads, silicon

carbide beads, mineral beads, or glass beads, and is preferably a calcium mineral, for example hydroxyapatite and/or tricalcium phosphate.

Solid formulations were prepared by mixing Zyderm®
 5 II (65 mg/mL collagen) or collagen-mPEG (63 mg/mL) with particulate hydroxyapatite and tricalcium phosphate (HA+TCP) and air drying to form a solid block containing 65% HA by weight. Optionally, blocks were heat-treated by heating at 75°C for 10 hours. The resulting blocks
 10 were hydrated in 0.13 M saline for 12 hours prior to testing.

On standing, it was observed that Zyderm®-HA+TCP (Z-HA) compositions separated into three phases, whereas PEG-collagen-HA+TCP (PC-HA) compositions remained single
 15 phase.

Each block was elongated by 5%, and its stress relaxation monitored for 1 minute after release. After this test, each block was subjected to constant elongation at a constant 1 cm/min until failure. The results
 20 are shown in Table 3:

Table 3: Mechanical Strength

| Sample | <u>Stress Relaxation</u> | | | <u>Constant Extension</u> | |
|--------------|--------------------------|----------------|-----------------|---------------------------|----------------------|
| | Peak Force | Constant Force | $t_{1/2}$ (min) | Rupture Force | Extension at Rupture |
| Z-HA (air) | 1.5 | 1.1 | 0.04 | 2.6 | 11.0% |
| | - | - | - | 2.6 | 15.3% |
| Z-HA (heat) | 1.5 | 1.1 | 0.06 | - | - |
| | 1.4 | 1.0 | 0.07 | 3.4 | 14.0% |
| PC-HA (air) | 2.6 | 1.8 | 0.06 | 5.5 | 12.3% |
| | 2.8 | 2.1 | 0.08 | 5.4 | 11.7% |
| PC-HA (heat) | 3.3 | 2.6 | 0.04 | 5.4 | 12.0% |
| | 3.6 | 2.7 | 0.06 | 5.4 | 20.3% |

40 All forces reported in newtons. Extension at rupture (strain) reported in percent extension.

The data demonstrate that collagen-polymer forms HA+TCP compositions exhibiting substantially greater tensile strength. Thus, one can prepare implant compositions with collagen-polymer which are substantially
5 stronger than compositions employing the same amount of non-conjugated collagen, or may reduce the amount of collagen-polymer employed to form a composition of equal strength.

WHAT IS CLAIMED:

1. A composition comprising collagen chemically conjugated to a synthetic hydrophilic polymer.
2. The composition of claim 1 wherein said collagen is type I, type II or type III.
3. The composition of claim 1 wherein said polymer is polyethylene glycol having a molecular weight of about 400 to about 20,000.
4. The composition of claim 1 wherein said synthetic hydrophilic polymer is bound to an available lysine residue on said collagen.
5. The composition of claim 4 wherein said polymer has a first end and a second end, said first end being bound to said lysine residue, and said second end being nonbound.
6. The composition of claim 5 wherein said synthetic hydrophilic polymer molecules are bound to 20-30% of said available lysine residues.
7. The composition of claim 1 wherein the ratio of collagen molecules to polymer molecules is about 1:1 to about 1:20.
8. The composition of claim 1, wherein said synthetic hydrophilic polymer has a first end and a second end; said first end being bound to a collagen molecule, and said second end being bound to a growth factor.
9. A composition suitable for augmentation of soft tissue, which comprises:

the composition of claim 1, in combination with a sufficient amount of a fluid pharmaceutically acceptable carrier.

5 10. The composition of claim 9 wherein said collagen is atelopeptide fibrillar collagen.

11. The composition of claim 9 wherein said polymer is monomethyl-polyethylene glycol having a
10 molecular weight of about 1,900 to about 8,000.

12. The composition of claim 9 wherein said polymer has a first end and a second end, wherein said first and second ends are bound to collagen to form a
15 crosslinked collagen-polymer conjugate, said composition having the form of a suspension of collagen-polymer conjugate particles in a pharmaceutically acceptable carrier.

20 13. The composition of claim 9 or 12 which further comprises a tissue growth-promoting amount of a growth factor selected from the group consisting of epidermal growth factor, transforming growth factor- β , or a hematopoietic factor.

25 14. The composition of claim 4 or 20 wherein said factor is chemically bound to said collagen by a covalent bond between said factor and the first end of said polymer, and a covalent bond between the second end
30 of said polymer and said collagen.

15. A composition suitable for augmentation of soft tissue, which comprises:

collagen;

35 a synthetic hydrophilic polymer having a first and second end, wherein said first and second ends comprise reactive groups capable of forming a covalent bond in

situ with an available lysine side chain present in collagen; and

a fluid pharmaceutically acceptable carrier in an amount sufficient to form an injectable composition.

5

16. The composition of claim 15 wherein said polymer comprises polyethylene glycol having an average molecular weight of about 400 to about 20,000.

10

17. The composition of claim 15 wherein said reactive groups comprise N-hydroxysuccinimide esters.

15

18. The composition of claim 15 wherein said composition comprises about 10 to about 100 mg/mL collagen and about 0.1 to about 30% synthetic hydrophilic polymer having reactive groups.

20

19. The composition of claim 18 wherein said composition comprises about 30 to about 80 mg/mL collagen and about 0.3 to about 10% synthetic hydrophilic polymer having reactive groups.

25

20. The composition of claim 15 wherein said collagen comprises fibrillar atelopeptide collagen.

21. The composition of claim 15 wherein said collagen comprises nonfibrillar collagen.

30

22. A composition suitable for repair of bone defects, which comprises:

the composition of claims 1, 8, 12 or 15, in combination with

a suitable particulate material; and

a sufficient amount of a fluid pharmaceutically acceptable carrier.

35

23. The composition of claim 22, wherein said particulate material is provided in sufficient amount to provide a rigid composition.

5 24. The composition of claim 22 or 23 wherein said collagen is reconstituted atelopeptide fibrillar collagen.

25. The composition of claim 22 or 23 wherein
10 said polymer is monomethyl-polyethylene glycol having a molecular weight of about 5,000.

26. The composition of claims 1, 22, or 25
wherein said polymer is chemically conjugated to 10-50%
15 of the available lysine residues on said collagen.

27. The composition of claim 22 or 23 wherein
said suitable particulate material comprises fibrillar
crosslinked collagen, gelatin beads, polytetrafluoro-
20 ethylene beads, silicone rubber beads, hydrogel beads,
silicon carbide beads, glass beads, hydroxyapatite
particles, tricalcium phosphate particles, or mixtures of
hydroxyapatite and tricalcium phosphate particles.

28. The composition of claim 22 or 23 wherein
said particulate material comprises hydroxyapatite
particles, tricalcium phosphate particles, or mixtures of
hydroxyapatite and tricalcium phosphate particles having
an average diameter of about 1 to 20 microns in diameter.

30

29. The composition of claims 8, 22 or 23 which
further comprises an effective amount of epidermal growth
factor, transforming growth factor- α , transforming growth
factor- β , transforming growth factor- β 1, transforming
35 growth factor- β 2, platelet-derived growth factor-AA,
platelet-derived growth factor-AB, platelet-derived
growth factor-BB, acidic fibroblast growth factor, basic

fibroblast growth factor, insulin-like growth factors, interleukins, colony stimulating factors, erythropoietin, nerve growth factor, interferons, and osteogenic factors.

5 30. A composition for replacing or augmenting cartilage, which composition comprises:

 collagen crosslinked with a hydrophilic synthetic polymer, said composition having a density of about 0.5 to about 1.5 g/cm³.

10

 31. An implant exhibiting reduced immunogenicity and tissue irritation, comprising:

 a solid implant, coated with a composition comprising collagen crosslinked with a synthetic
15 hydrophilic polymer.

 32. A method for preparing a collagen-polymer conjugate suitable for administration to mammals, which method comprises:

20 providing an aqueous mixture of collagen molecules;
 adding a solution of an activated polymer to form a reaction mixture, wherein said activated polymer comprises a hydrophilic polymer having a reactive group capable of forming a covalent bond with an available
25 lysine side chain present in said collagen molecules;
 and

 causing said polymer to form covalent bonds to said collagen molecules, forming a collagen-polymer conjugate.

30 33. The method of claim 32 wherein said activated polymer comprises two reactive groups, and said covalent bond formation crosslinks said collagen molecules.

35 34. The method of claim 32 wherein said hydrophilic polymer comprises polyethylene glycol having a molecular weight of about 400 to about 20,000.

35. The method of claim 34 wherein said reaction mixture contains activated polymer in an amount of about 0.1 to about 30% by weight.

5

36. The method of claim 32 which further comprises:

casting said reaction mixture into a predetermined shape during said crosslinking.

10

37. The method of claim 32 wherein said shape is a membrane or a tube.

38. The method of claim 32 which further comprises:

applying said reaction mixture to a solid support during said crosslinking.

39. The method of claim 38 wherein said solid support comprises a catheter.

40. The method of claim 38 wherein said solid support comprises a stress-bearing bone implant.

41. The method of claim 32 wherein said reaction mixture is vigorously agitated during said crosslinking, providing said collagen-polymer conjugate in the form of a particulate.

42. The method of claim 32 wherein said activated polymer solution further comprises an effective amount of a growth factor, wherein said solution is prepared by:

providing a solution of an activated polymer comprising two reactive groups;

adding said solution to a growth factor solution containing an effective amount of a growth factor; and

44

causing said polymer to form covalent bonds to said growth factor molecules, forming an activated polymer/growth factor conjugate solution.

5

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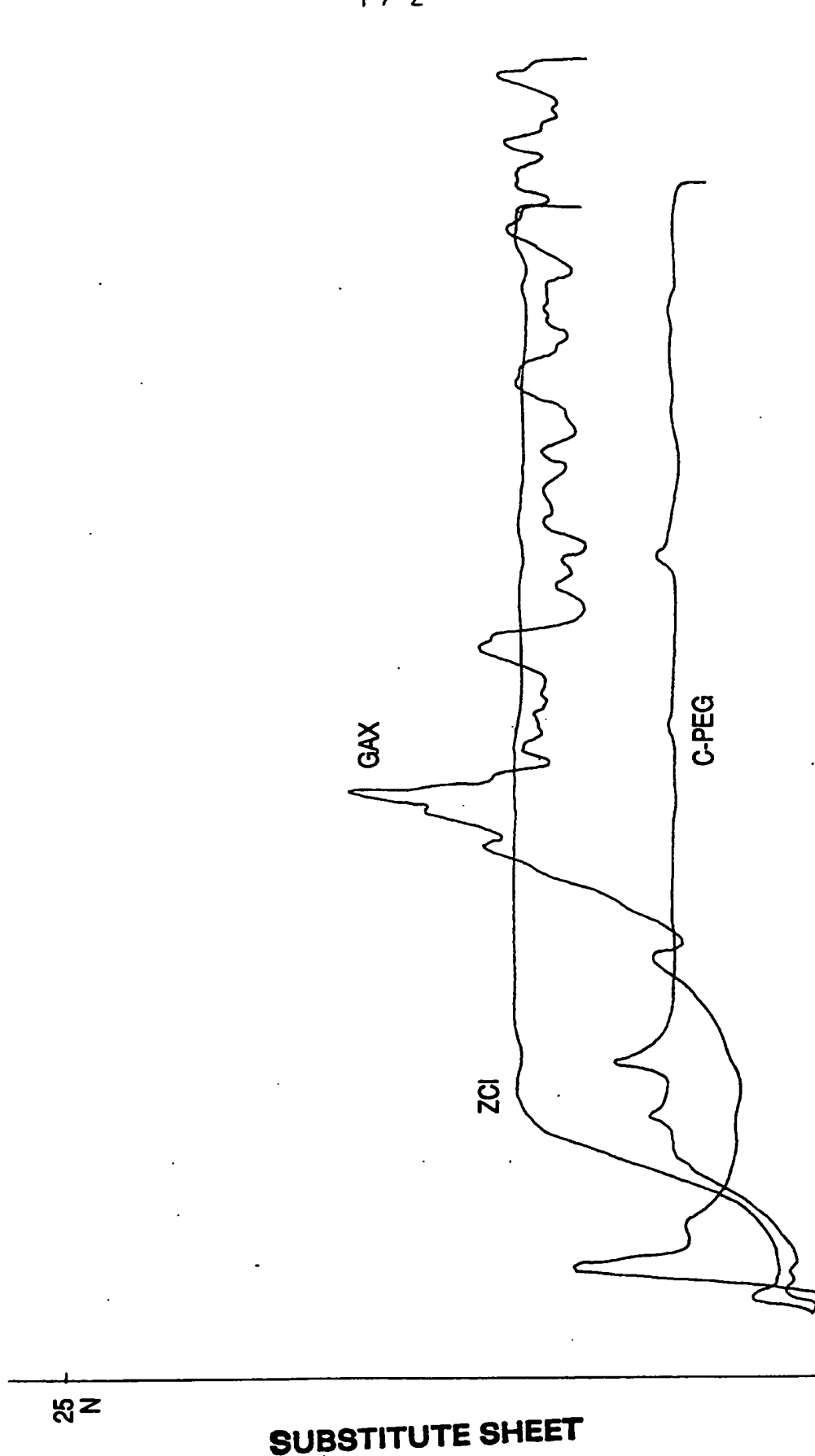


FIG. 1

SUBSTITUTE SHEET

2 / 2

RELEASE OF [¹²⁵I] -TGF- β 1 FROM TGF- β 1: DIFUNCTIONAL PEG: COLLAGEN SAMPLES

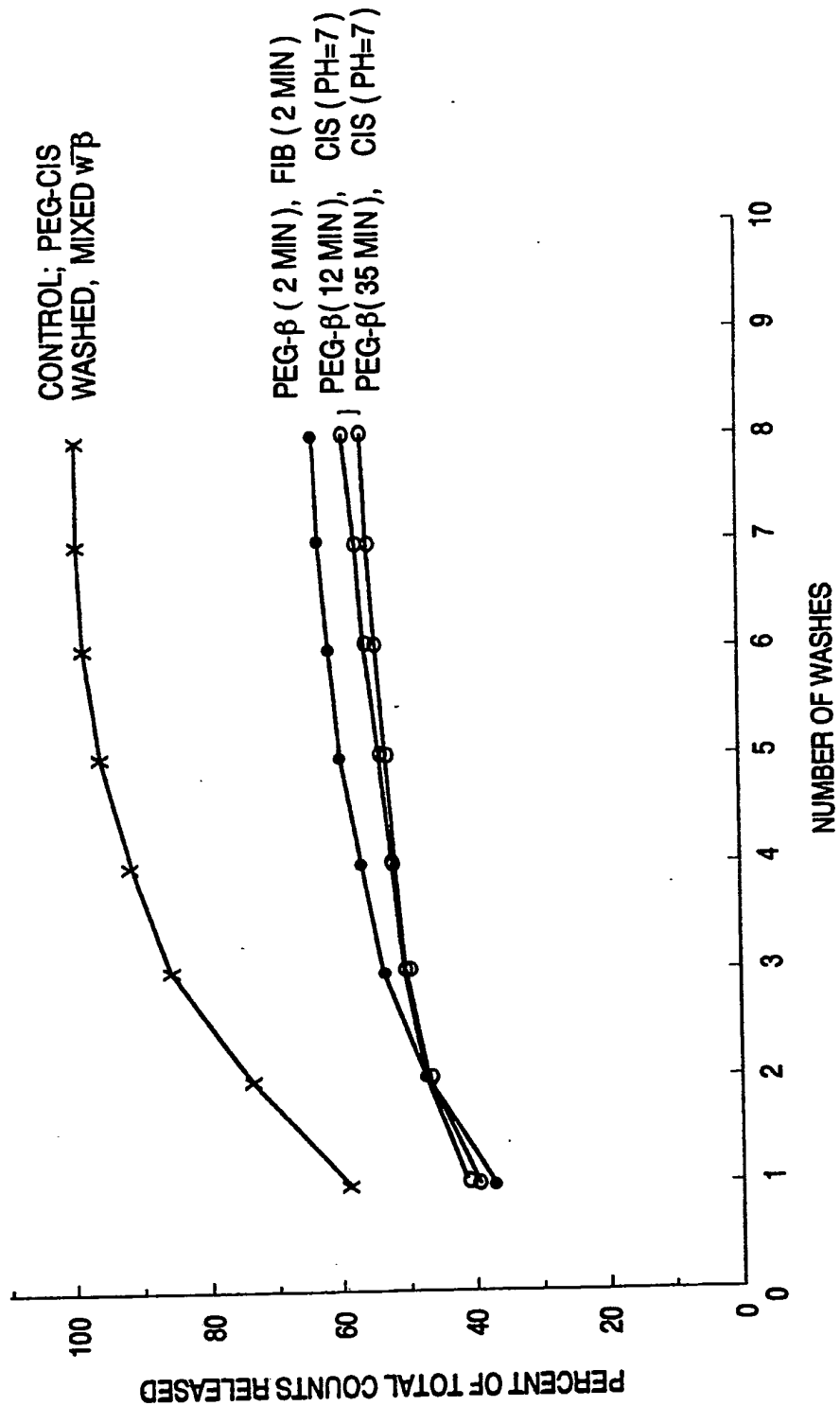


FIG. 2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 89/05351

| | | |
|---|---|---|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC | | |
| INT CL 5 C08G 83/00 C08H-1/00 C08J 3/00 C08L 89/00 A61K 37/12 US CL 525/54.1 530/356, 840 3/1 120/DIG 8 604/891 523/113 | | |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁷ | | |
| Classification System | Classification Symbols | |
| US CL | 525/54.1 530/356, 840 3/1 128/DIG 8 604/891 523/113 | |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸ | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ | | |
| Category [*] | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| X Y | Analytical Biochemistry Vol. 141, pp. 361-365, (1984) "Precipitation of Collagens by Poly- ethylene Glycols" (RAMSHAW). See the Experimental Procedures at page 361 and the Abstract. | 1-3, 7, 9-12, 14 1-16 |
| X Y | WO, A 87/04078 (BATEMAN) 16 July 1987 See page 5 (lines 26-32) for collagen materials. See page 6 (lines 13-27) for the water soluble polymers. See page 7 (lines 4-23) for use of growth factors. | 1-3, 7-9, 11-14 1-16 |
| Y | US, A 4,496,689 (MITRA) 29 January 1985 See the Abstract and the paragraph bridging column 5 to column 6 for the coupling mechanism. | 1-16 |
| Y P | US, A 4,847,325 (SHADLE) 11 July 1989 See the Abstract for the coupling mechanism. See Column 12 (lines 17-38) for the polymers. | 1-16 |
| <p>[*] Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search | | Date of Mailing of this International Search Report |
| 22 JANUARY 1990 | | 06 MAR 1990 |
| International Searching Authority | | Signature of Authorized Officer |
| ISA/US | | NATHAN M. NUTTER |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|---|----------------------|
| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| Y | WO,A 84/01106 (SPORN) 29 March 1984 See the Abstract and Examples for use of growth factors with other topical or systemic compositions. | 1-16 |
| A | US,A 3,619,371 (CROOK) 09 November 1971 | 1-16 |
| A | US,A 3,788,948 (KAGEDAL) 29 January 1974 | 1-16 |
| A | US,A 3,876,501 (HANUSHEWSKY) 08 April 1975 | 1-16 |
| A | US,A 3,960,830 (BAYER) 01 June 1976 | 1-16 |
| A | US,A 3,949,073 (DANIELS) 06 April 1976 | 1-16 |
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| A | US,A 4,412,989 (IWASHITA) 01 November 1983 | 1-16 |
| A | US,A 4,414,147 (KLÍBANOV) 08 November 1983 | 1-16 |
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| A | US,A 4,424,208 (WALLACE) 03 January 1984 | 1-16 |
| A | US,A 4,488,911 (LUCK) 18 December 1984 | 1-16 |
| A | US,A 4,495,285 (SHIMIZU) 22 January 1985 | 1-16 |
| A | US,A 4,557,764 (CHU) 10 December 1985 | 1-16 |
| A | US,A 4,563,350 (NATHAN) 07 January 1986 | 1-16 |
| A | US,A 4,582,640 (SMESTAD) 15 April 1986 | 1-16 |
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| A | US,A 4,678,468 (HIROYOSHI) 07 July 1987 | 1-16 |
| A | US,A 4,689,399 (CHU) 25 August 1987 | 1-16 |
| A | US,A 4,732,863 (TOMASI) 22 March 1988 | 1-16 |

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| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
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| A | US, A 4,766,106 (KATRE) 23 August 1988 | 1-16 |
| A | Journal of Free Radicals in Biology & Medicine Vol. 2 pp. 283-288, 1986. "Safety Evaluation of Free Radical Scavengers PEG-Catalase and PEG-Superoxide Dismutase" (VIAU) | 1-16 |
| A | American Journal of Veterinary Research Vol. 47, No. 6, June 1986. "Toxicologic studies of a conjugate of asparaginase and polyethylene glycol in mice, rats, and dogs" (VIAU) | 1-16 |
| A | J. Pharm. Pharmacol. Vol. 36, pp 354-355, January 1984. "Hypouricemic effect after oral administration in chickens of polyethylene glycol-modified uricase entrapped in liposomes" (NISHIDA) | 1-16 |
| A | Biochemical and Biophysical Research Communications Vol. 122, No. 2, July 31, 1984. "Ester Synthesis Catalyzed By Polyethylene Glycol-Modified Lipase In Benzene" (INADA) | 1-16 |
| A | Biochemical and Biophysical Research Communications Vol. 121, No. 1, May 31, 1984. "A Chemical Modification to Make Horseradish Peroxidase Soluble and Active in Benzene" (TAKAHASHI) | 1-16 |
| A | Cancer Biochem. Biophys. Vol. 7, pp 175-186. 1984. "Cancer Therapy With Chemically Modified Enzymes. I. Antitumor Properties of Polyethylene Glycol-Asparaginase Conjugates" (ABUCHOWSKI) | 1-16 |
| A | Clin. Exp. Immunol. Vol. 48 pp 273-278. "Immunological effects of native and polyethylene glycol-modified asparaginases from <u>Vibrio Succinogenes</u> and <u>Escherichia coli</u> in normal and tumour-bearing mice" (BENDICH) | 1-16 |

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